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International Journal of Pharmaceutics 314 (2006) 37–45
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Influence of modified cyclodextrins on solubility and percutaneous absorption of celecoxib through human skin

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Received 27 September 2005; received in revised form 31 January 2006; accepted 2 February 2006

Abstract

We evaluated the ability of two modified cyclodextrins, hydroxypropyl-β-cyclodextrin (HP-β-Cyd) and 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-Cyd), to influence the percutaneous absorption through isolated human stratum corneum and epidermis (SCE) of celecoxib (CCB). Previous studies demonstrated that DM-β-Cyd includes the drug, producing a significant increase of water solubility (0.5 mg/ml at 25 °C) and dissolution rate of CCB. In this work chemical-physical characterization studies were performed to evaluate the ability of HP-β-Cyd to include CCB. We showed that only an external interaction could exist between CCB and HP-β-Cyd that positively influences the water solubility of the drug (0.12 mg/ml at 25 °C for CCB-HP-β-CyD system and 4.12×10^{-3} mg/ml at 25 °C for free CCB). In vitro percutaneous experiments were performed using samples in solution and in suspension containing different Cyd concentrations. Both HP-β-Cyd and DM-β-Cyd enhanced drug flux through SCE by means of an increase of dissolution rate of the drug as well as a direct action on the stratum corneum (SC). Histological analysis of treated SCE showed a protective effect of the two Cyds towards an invasive action shown by CCB on SC.
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Keywords: Celecoxib; Modified cyclodextrins; In vitro percutaneous studies; Isolated human skin; Histological analysis

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most active agents used in inflammatory diseases. They act by inhibiting the enzymatic activity of cyclooxygenase (COX), showing not only therapeutic effects but also side effects.

Celecoxib (CCB) is the first synthesized NSAID able to selectively inhibit COX-2 activity. For this reason, CCB shows high efficacy in the treatment of osteoarthritis and rheumatoid arthritis (Simon et al., 1998; Annoni and Strumia, 2000 and references therein), but no gastrolesivity or interference with platelet function was observed at therapeutical concentrations. However, different authors (Marques et al., 2003; Goeschke and Braathen, 2004; Yang et al., 2004) reported the appearance of benign skin damage with generalized pustular exanthema in patients treated systemically with CCB, due to the presence of sulphonamide group in the molecule. Topical application of CCB could increase the presence of the drug locally, reducing, in the mean time, the risk of systemic skin toxicity as a result of a reduced dose.

CCB is a 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide and shows high apolar characteristics (Fig. 1). The drug is insoluble in water and could show per se poor percutaneous absorption properties. Moreover, the protective role played by the stratum corneum could make the topical application of CCB ineffective. Cyclodextrins (Cyds) could influence the percutaneous absorption of CCB by both a solubilizing action on the drug (Felton et al., 2002; Rode et al., 2003; Babu and Pandit, 2004), thus increasing its availability at the absorption site, and by an interaction with the free lipids present in the stratum corneum (Swartzendruber et al., 1987), improving transdermal penetration of CCB (Vitória et al., 1997; Loftsson and Masson, 2001; Ventura et al., 2001).

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doi:10.1016/j.ijpharm.2006.02.006

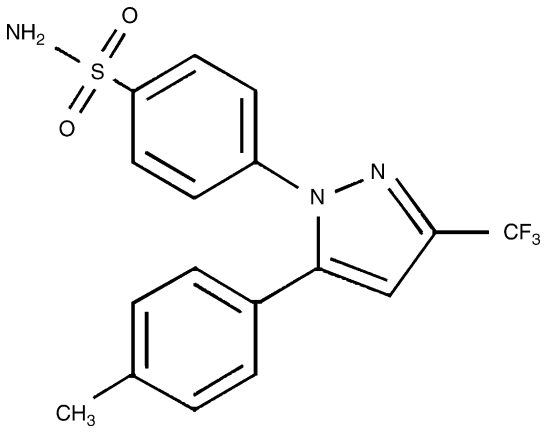

Fig. 1. Chemical structure of CCB.

We recently prepared and characterized the inclusion complex of CCB with 2,6-dimethyl-β-CyD (DM-β-Cyd) (Ventura et al., 2005). DM-β-Cyd is able to include CCB, increasing the water solubility and dissolution rate of the drug. Permeation of CCB through the CaCo-2 cell monolayer was significantly increased in the presence of the macrocycle.

In this work we evaluated the ability of DM-β-Cyd and hydroxypropyl-β-Cyd (HP-β-Cyd) to influence the percutaneous absorption of CCB through isolated human stratum corneum and epidermis (SCE). In vitro permeation experiments were performed using Franz cells. Characterization studies in the solid state and in aqueous solution were performed, compared to CCB-DM-β-Cyd inclusion complex, to evaluate the ability of HP-β-Cyd as a complexing carrier for CCB. Histological analysis of isolated human SCE treated with the two complexes, free Cyds and CCB alone, were performed to evaluate the different mechanism by which Cyds act.

2. Materials and methods

2.1. Materials

CCB was obtained by repeated extractions with methanol from a marketed capsule formulation (Solexa®; Pfizer) and the purity (99%) was assayed by ^{1}H NMR, HPLC and elemental analysis (Calcd. for $C_{17}H_{14}F_3N_3O_2S$: C, 53.54; H, 3.7; N, 11.01; S, 8.39; found: C, 52.99; H, 3.81; N, 10.84; S, 8.91). HP-β-CyD, with 0.6 degree average substitution, was kindly provided by Roquette Italia (Cassano Spinola, Italy). DM-β-Cyd was purchased from Cyclolab R&D Laboratory (Budapest, Hungary) and used without further purification.

All other chemicals and solvents were of analytical reagent grade and obtained from Sigma–Aldrich (Milano, Italy). Deionized double-distilled water was used throughout the study.

2.2. Preparation of the CCB-Cyds solid samples

CCB-DM-β-Cyd and CCB-HP-β-Cyd solid samples were prepared by the freeze-drying method as described in our previous paper (Ventura et al., 2005). Briefly, a water/methanol solution (50:50, v/v) containing DM-β-Cyd or HP-β-Cyd was added to an excess amount of solid CCB. After stirring at room temperature for 2 days, the suspensions were filtered through a 0.45 μm Nylon filter (Millipore, Bedford, U.S.A.) and the filtrates were freeze-dried using the Modulyo 4 K system (Edwards, Crawley, U.K.). The obtained CCB-DM-β-Cyd solid sample (10 mg) was solubilized in methanol (5 ml) and analyzed by HPLC to determine the drug-Cyd molar ratio. CCB-HP-β-Cyd solid sample (10 mg) was suspended in methanol (5 ml) and stirred for 2 h. The suspension was filtered and the solution was analyzed for molar ratio.

CCB-Cyds physical mixtures were prepared in 1:2 molar ratio by simple mixing in a mortar for 15 min.

2.3. Differential scanning calorimetry (DSC)

DSC scans were recorded on a Mettler DSC 12E (Mettler Toledo Italia, Milano, Italy); equipped with a Haake thermocryostate mod. D8-G (Haake, Karlsruhe, Germany). A Mettler TA89E and FP89 system software was used for data acquisition. Indium was used to calibrate the instrument. Each sample was scanned at a speed of 10 °C/min in the 30–300 °C temperature range.

2.4. Circular dichroism (CD) spectra

CD spectra were performed on a Jasco J-600D recording spectropolarimeter (Jasco, Inc., Easton, MD, USA). CCB alone or in the presence of different concentrations of both Cyds (1:10 or 1:100 molar ratio) were solubilized in a water/methanol solution (70/30, v/v) and stirred before the analysis for 12 h.

2.5. ^{1}H NMR studies

^{1}H NMR spectra were recorded, at a probe temperature of 303 °K, on a VARIAN Unity Inova Instrument at 200 MHz (Varian, Palo Alto, CA, USA), For the analysis, CCB (0.4 mg) and the Cyds (1:1 and 1:2 molar ratios for CCB-DM-β-Cyd; 1:1, 1:2 and 1:3 molar ratios for CCB-HP-β-Cyd) were poured into vials and added to 1 ml of D_2O/CD_3OD solution (50/50, v/v). After stirring for 24 h, 0.7 ml of these solutions were submitted to analysis. Free CCB, DM-β-Cyd and HP-β-Cyd were solubilized in the same solvent mixture. No internal standards were added to the samples due to their interaction with Cyd cavity, the residual sign of CD_3OD at 3.3 ppm was used as reference.

2.6. Water solubility and dissolution rate determination

Water solubility of CCB-HP-β-Cyd solid samples, compared to free CCB and CCB-DM-β-Cyd, was determined by suspending excess amounts of each sample in 2 ml of water and stirring at room temperature for 2 days. The suspensions were then filtered (0.45 μm Nylon Millipore filter) and analyzed by HPLC.

Dissolution rates of the same samples were carried out according to the USP 25th paddle method. An amount of 50 mg of free CCB or a corresponding amount in complexes and physical mixtures were suspended in 900 ml of water and stirred at 100 rpm at 37 ± 0.5 °C. At fixed time intervals the concentration

of CCB in solution was assayed by HPLC. The medium was reconstituted with fresh water and the data were corrected for the operated dilution. The experiments were carried out in triplicate.

Free CCB was freeze-dried before being used by the same procedure employed to prepare CCB-DM-β-Cyd solid sample.

2.7. Solubility studies

Solubility phase diagrams of CCB-Cyd systems were obtained by the Higuchi and Connors' method (Higuchi and Connors, 1965). Excess amounts of CCB were added to phosphate buffer solutions (pH 7) containing various concentrations of HP-β-Cyd or DM-β-Cyd ($0–38 \times 10^{-3}$ M) and shaken for 2 days at 25 °C. The suspensions were filtered through a 0.45 μm Nylon Millipore filter and the CCB concentration was determined by HPLC.

2.8. HPLC analyses

HPLC analyses were performed at room temperature (25 °C) using a 1050 Hewlett Packard apparatus (Hewlett Packard, Milano, Italy) on a 5 μm Hypersil ODS cartridge (125 mm × 4 mm i.d.) (Hewlett Packard) equipped with a 5 μm Hypersil 100 RP-18 guard cartridge (4 mm × 4 mm i.d.) (Hewlett Packard) and eluted isocratically with acetonitrile/water (55/45, v/v). The flow rate was fixed at 1 ml/min and UV light at 252 nm was used for detection.

2.9. Preparation of samples for permeability studies

For permeation experiments, CCB-Cyd solid samples were prepared by a freeze-drying method. CCB (1 mg) was solubilized in 500 μl of methanol and added to aqueous solutions (2 ml) containing various concentrations of DM-β-CyD or HP-β-Cyd (1.5%, 3%, 5% and 10%, w/v). The obtained water/methanol solutions were stirred for 2 h, than freeze-dried. Free CCB was also freeze-dried before being used.

Water (1 ml) was added to freeze-dried CCB-CyD samples and to free CCB (1 mg) and stirred at room temperature for 12 h before the permeation experiments. After this time some samples were in suspension (free CCB, the samples prepared in the presence of 1.5% (w/v) of both Cyds and those prepared in the presence of 3% and 5% (w/v) HP-β-Cyd). All the remaining samples were in solution (the sample prepared in the presence of 10% (w/v) HP-β-Cyd and the samples prepared with 3%, 5% and 10% (w/v) DM-β-Cyd).

2.10. In vitro percutaneous experiments

Samples of adult human female skin (mean age 42 ± 7) were obtained from abdominal reduction surgery. Subcutaneous fat was carefully trimmed and the stratum corneum and the epidermis (SCE) were separated from the dermis in accordance with the procedure described by Kligman and Christophers (1963). SCE membranes were dried in a desiccator at approximately 25% relative humidity and stored at 4 °C until used, in accordance with Swarbrick et al. (1982). Samples of dried SCE were dehydrated by immersion in distilled water at room temperature for 1 h before being mounted in Franz-type diffusion cells (LGA, Berkeley, CA, USA) with the stratum corneum (SC) side up. The area of SCE available for diffusion was 0.75 cm^2. The donor compartment was covered with Parafilm® (American National Can, Greenwich, CT, USA) in order to achieve occlusive conditions. The receptor was filled with 4.5 ml of water/ethanol solution (50/50, v/v) to ensure pseudo-sink conditions by increasing CCB solubility in the receptor phase. The receptor fluid was constantly stirred with a small magnetic stirring bar to ensure homogeneity. The apparatus was thermostated at 37 ± 0.5 °C throughout the experiment. After the application of 200 μl of the previously described suspensions or solutions, samples from the receptor phase (200 μl) were withdrawn at predetermined time intervals (0, 0.4, 1, 2, 3, 4, 5, 6, 22 and 24 h) and the CCB concentration was determined by HPLC. Each aliquot withdrawn was replaced with an equal volume of receptor phase.

2.11. Data calculation

The concentration of CCB in the receptor compartment was corrected for dilution due to the sampling procedure. Data were analyzed by linear regression of the cumulative amount of the drug permeated for skin surface unity (Qs) versus time in the steady-state range. Flux (Js) is represented by the slope of the regression lines. The lag time (T_L) was determined from the *x*-intercept values of the regression lines. All the obtained permeation profiles were determined six times in different experiments and the mean values ± standard deviations were calculated.

2.12. Histological analysis

Histological analysis was performed on scrap of SCE coming from the same batch used for in vitro permeation studies and treated with free CCB and CCB-CyD samples prepared as described above. SCE was mounted on Franz cells and 200 μl of free CCB, CCB-DM-β-Cyd and CCB-HP-β-Cyd systems, containing 3% (w/v) of Cyd, and the Cyds alone (3%, w/v), were applied in the donor compartment. The receptor compartment was filled with saline. To avoid the supersaturation of CCB in this phase, samples were withdrawn at short time intervals (20 min) and immediately replaced with an equal volume of saline.

At the end of the experiment (24 h) the SCE samples were fixed with formalin solution (10%, w/v in phosphate buffer solution, pH 7.4) and embedded in paraffin. The 5 μm thick sections obtained using a Reichert-Jung 2050 microtome (2050 Supercut Reichert-Jung, Leica Instruments, Wetzlar, Germany) were stained with hematoxylin-eosine. Sections were observed and photographed with a Leika microscope DM-LB (Leika, Heidelberg, Germany).

3. Results

3.1. Physical–chemical characterization

CCB-HP-β-Cyd and CCB-DM-β-Cyd solid samples obtained by freeze-drying showed a 1:2 molar ratio. The

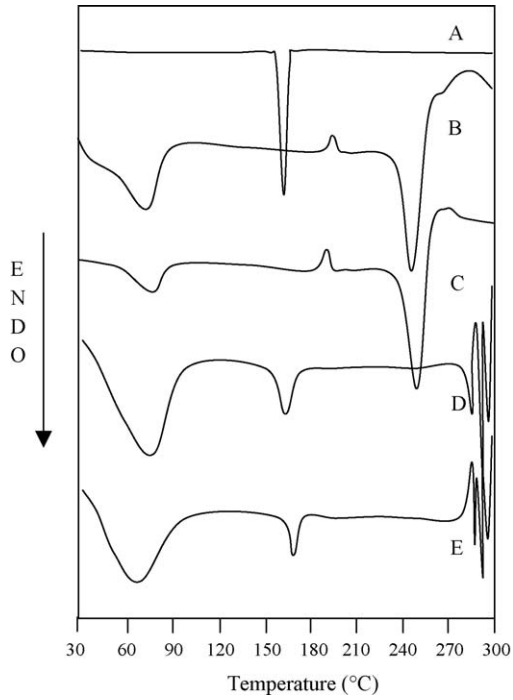

Fig. 2. DSC thermograms of CCB-Cyd solid systems: (A) CCB alone; (B) CCB-DM-β-Cyd physical mixture; (C) CCB-DM-β-Cyd solid sample; (D) CCB-HP-β-Cyd physical mixture; (E) CCB-HP-β-Cyd solid sample.

obtained systems were characterized in the solid state by DSC analysis compared to physical mixtures in the same molar ratio. The thermograms in Fig. 2 show a different trend for the two solid samples. The calorimetric curve of CCB-HP-β-Cyd sample was similar to the thermogram of the physical mixture, showing the CCB fusion peak at 160 °C (Ventura et al., 2005; Chawla et al., 2003). As concerns the CCB-DM-β-Cyd sample, we observed a disappearance of the drug fusion peak and the appearance of a new peak at 250 °C. A similar trend was observed for CCB-DM-β-Cyd physical mixture (Ventura et al., 2005).

Aqueous solution studies were performed to investigate the existence of interactions between CCB and HP-β-Cyd in water. The free drug did not show CD bands and no induced CD band was registered in the presence of different concentrations of HP-β-Cyd.

^{1}H NMR studies showed no shifts for CCB protons in the presence of HP-β-Cyd (1:1 and 1:2 molar ratio) (data not shown). Increasing the HP-β-Cyd molar ratio (1:3), only the H-4 proton of the pyrazole nucleus was shifted upfield ($\Delta\delta = -0.021$ ppm), probably as a result of a shielding effect produced by oxygen atoms of hydroxypropyl groups of a macrocycle close to H-4 proton. No shift was detected for internal protons of HP-β-Cyd because they overlap other macrocycle protons.

Different results were previously observed for CCB-DM-β-Cyd system (Ventura et al., 2005). Two CD induct bands appeared, one negative centered at 240 nm and the other one positive at 280 nm (data not shown). ^{1}H NMR studies showed upfield shifts for H-3 and H-5 Cyd protons and downfield shifts for all aromatic protons of CCB as a result of the inclusion

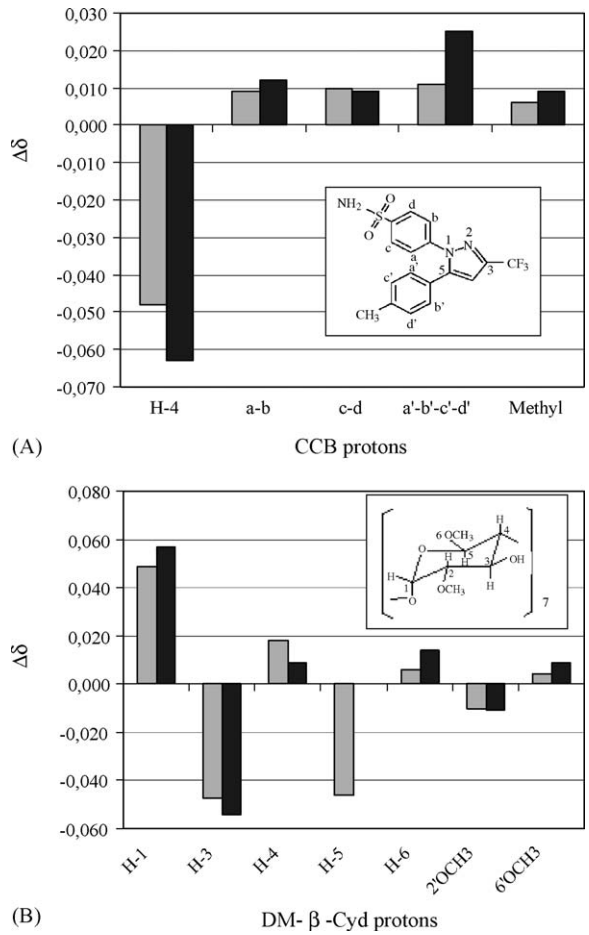

Fig. 3. Shift of CCB protons in the presence of DM-β-Cyd (A) and of DM-β-Cyd protons in the presence of CCB (B): (▒) 1:1 and (■) 1:2 drug-Cyd molar ratio. $\Delta\delta = \delta$complex $- \delta$free.

(Fig. 3A and B). A significant upfield shift was observed for the H-4 proton of CCB, which could be due to the association of this proton with the methoxy oxygen atoms of DM-β-Cyd, rich in π electrons. Nuclear overaus experiments seemed to confirm a 1:1 CCB-DM-β-Cyd stoichiometry (Ventura et al., 2005).

Even if no inclusion occurs between CCB and HP-β-Cyd we cannot exclude the presence of a no-inclusion interaction between the two components able to influence water solubility of CCB (Loftsson et al., 2002). For this reason, we performed solubility studies on the CCB-HP-β-Cyd system, compared to the CCB-DM-β-Cyd inclusion complex, using Higuchi and Connors' method (1965). The isotherms obtained at 25 °C are shown in Fig. 4.

A different trend was observed for the two systems. A_L type isotherm was observed for the CCB-HP-β-Cyd system with a slope value less than unity, showing the presence in solution of a complex with 1:1 stoichiometry (Higuchi and Connors, 1965). A positive curvature (Ap type isotherm) was observed for the CCB-DM-β-Cyd system, showing the presence in solution of two complexes with 1:1 and 1:2 CCB-DM-β-Cyd molar ratio (Ventura et al., 2005). The stability constants (K) for 1:1 CCB-HP-β-Cyd and 1:1 and 1:2 CCB-DM-β-Cyd systems, determined using Higuchi and Connors' equation (1965), are reported

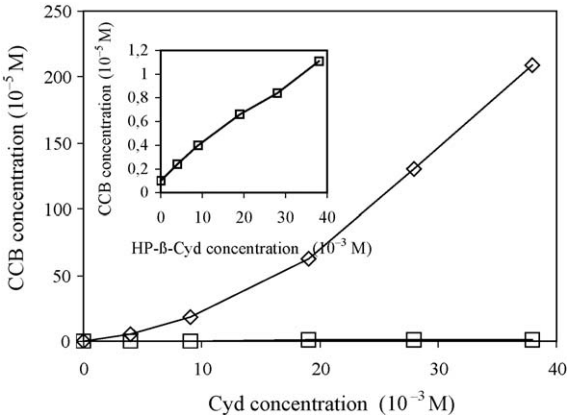

Fig. 4. Solubility phase diagrams of the CCB-Cyd systems at 25 ± 0.5 °C: (□) CCB-HP-β-Cyd system; (◊) CCB-DM-β-Cyd system.

Table 1
Stability constant values ($K_{1:1}$ and $K_{1:2}$) determined for CCB-Cyd systems in phosphate buffer solution (pH 7)

CCB-Cyd systems	$K_{1:1}$ (M^{-1})	$K_{1:2}$ (M^{-1})
CCB-DM-β-Cyd	9004.83	141.20
CCB-HP-β-Cyd	190.20	–

in Table 1. Higher $K_{1:1}$ value (9004 M^{-1}), with respect to $K_{1:2}$ value (141 M^{-1}) was observed for CCB-DM-β-Cyd complexes. The CCB-HP-β-Cyd system showed a lower $K_{1:1}$ value with respect to the CCB-DM-β-Cyd inclusion complex.

DM-β-Cyd was able to solubilize higher amounts of CCB (0.5 mg/ml and 4.12×10^{-3} mg/ml at 25 °C, respectively for CCB-DM-β-Cyd inclusion complex and free drug) compared to HP-β-Cyd (0.12 mg/ml at 25 °C). The solubility increase of CCB significantly influences its dissolution rate (Fig. 5) and a complete dissolution of the CCB-DM-β-CyD inclusion complex was observed within 30 min. In the case of the CCB-HP-β-Cyd system, only 15% (w/w) of CCB was dissolved within 180 min.

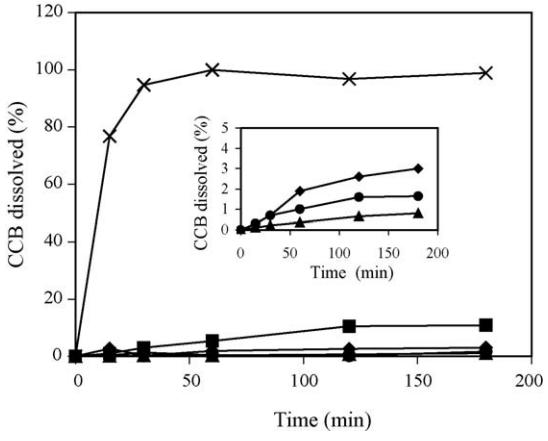

Fig. 5. Dissolution profiles of the CCB-Cyd systems: (▲) CCB alone; (●) CCB-HP-β-Cyd physical mixture; (♦) CCB-DM-β-Cyd physical mixture; (■) CCB-HP-β-Cyd complex; (×) CCB-DM-β-Cyd inclusion complex.

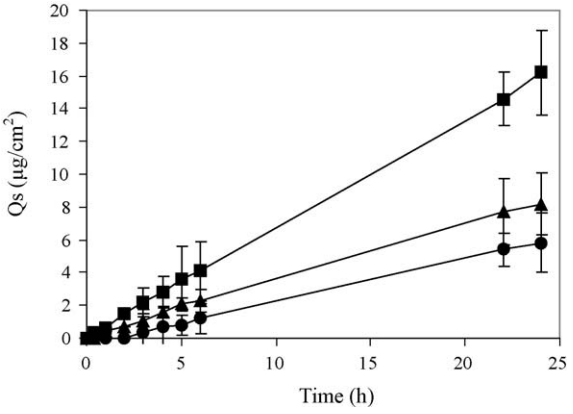

Fig. 6. Permeation profiles through human SCE of CCB alone or in the presence of DM-β-Cyd and HP-β-Cyd at 1.5% (w/v) concentration: (●) CCB alone; (▲) CCB-HP-β-Cyd system; (■) CCB-DM-β-Cyd complex.

3.2. In vitro percutaneous studies

The in vitro permeability studies were carried out using CCB-Cyd samples in solution and in suspension to evaluate the ability of Cyds to increase CCB permeation through the SCE by means of a drug solubility increase and/or a direct action on the SC.

The Qs of CCB permeated through the SCE as free drug or in the presence of the lowest DM-β-Cyd and HP-β-Cyd concentration (1.5%, w/v) are shown in Fig. 6, as a function of time.

Both Cyds influenced CCB permeation through the SCE. In the presence of DM-β-Cyd, CCB Js increases by about 2.5 times in comparison with CCB alone (Table 2). A significant reduction of T_L was also observed, from about 2 h for free CCB, to about 35 min in the presence of DM-β-Cyd. HP-β-Cyd produced no significant variation of CCB Js, however, a significative reduction of T_L was observed.

The permeation profiles obtained increasing DM-β-Cyd and HP-β-Cyd concentrations are shown in Fig. 7A–B and the permeation parameters are summarized in Table 2.

At 3% (w/v) Cyds the cumulative amount of CCB permeated within 24 h was about 42, 36 and 6 μg/cm^2, respectively for CCB-DM-β-CyD, CCB-HP-β-CyD and CCB alone. The drug permeation slightly increased again at 5% (w/v) concentration

Table 2
Percutaneous permeation parameters[a] of free CCB or in the presence of different concentrations (%, w/v) of DM-β-CyD and HP-β-CyD in water solution at 37 ± 0.5 °C

Compounds	Qs[b] (μg/cm^2)	T_L (h)	Js (μg/cm^2/h)
CCB	5.8 ± 1.8	1.81 ± 0.71	0.261 ± 0.06
CCB-DM-β-CyD (1.5%)	16.9 ± 2.6	0.63 ± 0.20	0.665 ± 0.09
CCB-DM-β-CyD (3%)	42.3 ± 3.0	0.21 ± 0.08	1.790 ± 0.07
CCB-DM-β-CyD (5%)	46.4 ± 4.0	0.13 ± 0.31	1.861 ± 0.19
CCB-DM-β-CyD (10%)	10.2 ± 2.6	1.62 ± 0.43	0.454 ± 0.07
CCB-HP-β-CyD (1.5%)	8.2 ± 1.9	0.59 ± 0.15	0.339 ± 0.12
CCB-HP-β-CyD (3%)	36.2 ± 3.9	0.42 ± 0.22	1.523 ± 0.26
CCB-HP-β-CyD (5%)	40.4 ± 4.5	0.24 ± 0.16	1.650 ± 0.16
CCB-HP-β-CyD (10%)	21.3 ± 4.1	1.02 ± 0.29	0.906 ± 0.08

[a] Each value is the average ± S.D. of six different experiments.
[b] Cumulative concentration for unity of surface after 24 h.

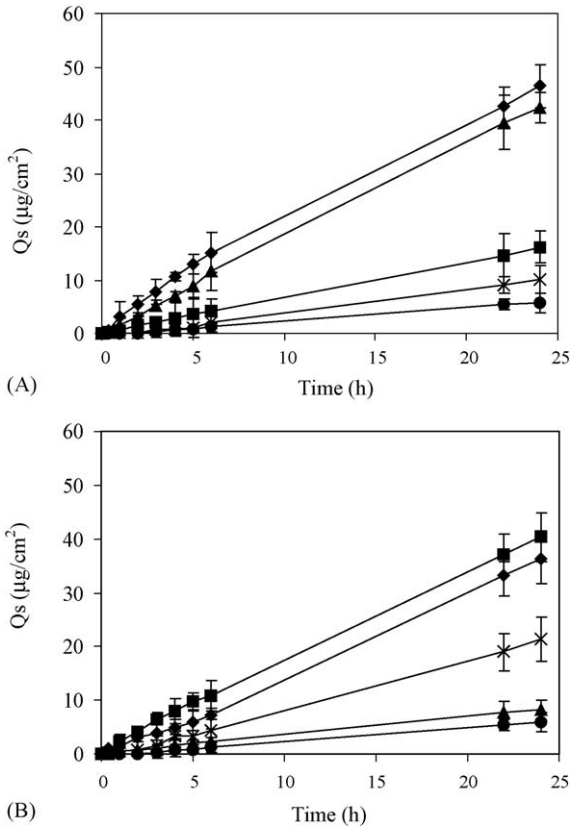

Fig. 7. (A) Permeation profiles through human SCE of CCB alone or in the presence of different concentrations of DM-β-Cyd: (●) CCB alone; (■) CCB + 1.5% DM-β-Cyd; (▲) CCB + 3% DM-β-Cyd CCB; (♦) CCB + 5% DM-β-Cyd; (×) CCB + 10% DM-β-Cyd. (B) Permeation profiles through human SCE of CCB alone or in the presence of different concentrations of HP-β-Cyd: (●) CCB alone; (▲) CCB + 1.5% HP-β-Cyd; (♦) CCB + 3% HP-β-Cyd CCB; (■) CCB + 5% HP-β-Cyd; (×) CCB + 10% HP-β-Cyd.

of both Cyds, while a consistent decrease was observed at the highest Cyd concentration (10%, w/v) (10.2 and 21.3 μg/cm^2, respectively for CCB-DM-β-CyD and CCB-HP-β-CyD).

In an attempt to clarify the effect of the two Cyds on SCE permeability to CCB, we performed histological analysis of the SCE previously treated with free CCB and Cyds and with CCB in the presence of DM-β-Cyd and HP-β-Cyd in 3% (w/v) concentration. The obtained photomicrographs are shown in Fig. 8.

We observed an invasive action of free CCB water suspension on SC (photo b) that caused the breaking of the horny thin plates. No influence was observed at the level of the spinosum layer that remained compact. In the presence of free DM-β-Cyd SC appeared flaked, with separation of corneocyte layers (photo c). A less invasive effect was exerted by free HP-β-Cyd (photo d), in the presence of which SC appeared similar to the control (photo a), even if a little separation of horny lamellae was present. No particular influence seemed to be exerted by the two Cyds on the epidermal layer.

Comparable results were obtained for SCE samples treated with the two complexes. We observed a separation of corneocytes layers and an imbibition of the epidermal layer in both cases. The CCB-DM-β-Cyd inclusion complex showed a less injurious action on SC with respect to free CCB and DM-β-Cyd (photo e), evidencing a protective effect of the macrocycle towards the invasive action shown by the free drug.

4. Discussion

4.1. Physico-chemical characterization

Solid state and solution studies evidenced the inability of HP-β-Cyd to include CCB into its cavity. In fact, a new endothermic peak did not appear in the DSC thermogram and no variation on spectroscopic characteristic of CCB was observed in the presence of HP-β-Cyd. The presence of hydroxypropyl groups probably produced a steric barrier that hinders the inclusion of the CCB molecule. In this way, by the freeze-drying method, only a solid dispersion was obtained. Different considerations can be made for the CCB-DM-β-Cyd systems. DSC curves, in fact, showed a new endothermic peak at a higher temperature with respect to the melting peak of free CCB that evidenced the ability of DM-β-Cyd to interact with CCB. However, the calorimetric trend of the CCB-DM-β-Cyd physical mixture, similar to that of the solid sample, does not allow us to confirm that the freeze-drying method produces a solid inclusion complex. Only a physical mixture could be obtained, which complexed during heating (Ventura et al., 2005). Solution studies confirmed the high affinity of the apolar cavity of DM-β-Cyd for the apolar CCB molecule. The presence of two CDI bands, the downfield shifts of the aromatic CCB protons and the upfield shifts of internal DM-β-Cyd protons evidenced a perturbation of the microenvironment polarity of CCB and of DM-β-Cyd as a result of complexation.

Higuchi and Connors' method was employed to evaluate the stoichiometry of the complexes. DM-β-Cyd is able to form with CCB two complexes at different stoichiometries; in particular, the obtained $K_{1:1}$ and $K_{1:2}$ values demonstrate that 1:1 complex was formed more easily than that at 1:2 molar ratio. We hypothesized the presence in solution of a 1:1 inclusion complex and a superficial or external interaction of CCB with the second Cyd molecule. The very low $K_{1:1}$ value observed for the CCB-HP-β-Cyd system was a result of the absence of an internal interaction between the two components.

4.2. In vitro percutaneous studies

The in vitro permeation studies of CCB through SCE demonstrated a similar trend in the presence of both Cyds considered. At first an increase of CCB permeation was observed with the increase of the Cyd concentration, but at the highest Cyd concentration a significative reduction of CCB Js was observed. This trend was related to two different factors: (i) a rapid dissolution rate of the drug when in the presence of Cyds instead of free drug and (ii) a probable direct action of the two Cyds on SC that destabilizes its protective role. To evaluate both contributions to the increase of CCB permeation through the SCE we performed in vitro studies using both in suspension and in solution CCB-Cyd samples.

At the lowest Cyd concentration (1.5%, w/v) both CCB-DM-β-Cyd and CCB-HP-β-Cyd samples were in suspension,

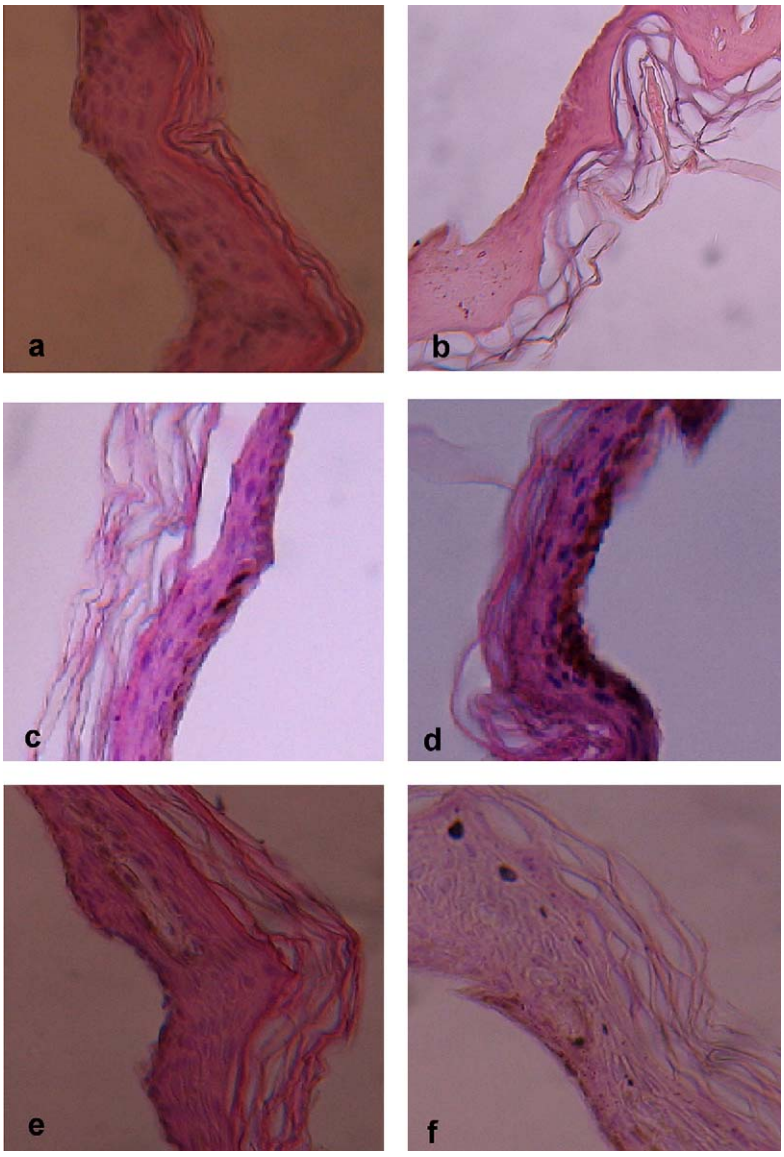

Fig. 8. Photomicrographs of SCE treated with free CCB and modified Cyds and CCB-Cyd systems: (a) control; (b) free CCB; (c) free DM-β-Cyd (3%, w/v); (d) free HP-β-Cyd (3%, w/v); (e) CCB in the presence of DM-β-Cyd (3%, w/v); (f) CCB in the presence of HP-β-Cyd (3%, w/v).

in this case the influence exerted by both Cyds on CCB Js could be exploited on the basis of a faster dissolution of CCB-Cyd systems with respect to the free drug. In fact, a more rapid dissolution can enhance CCB availability at the skin surface. This fact produced both faster (T_L reduction) and greater (Qs increase) permeation with respect to free CCB. On this basis, the low dissolution observed for the CCB-HP-β-Cyd system justifies the scarcely significative permeation of CCB observed for this system, with respect to the CCB-DM-β-Cyd inclusion complex.

Not only does dissolution play an important role in the percutaneous absorption of CCB from CCB-Cyd systems, but also K_c values are relevant. In fact, considering the great difference of dissolution rate observed for the two systems, significant differences were expected in the amount of CCB permeated through SCE from CCB-HP-β-Cyd and CCB-DM-β-Cyd complexes. However, CCB Js observed in the presence of DM-β-Cyd was only twice as high as that observed with HP-β-Cyd. Considering that Cyds permeate lipophilic biomembranes with considerably difficulty (Tanaka et al., 1995; Gerloczy et al., 1988) and that the hydrated drug/Cyd complexes are unable to permeate lipophilic biological membranes (Loftsson and Bodor, 1995; Rajewski and Stella, 1996), only the solubilized free drug can cross SCE. Thus, the highest K_c values observed for the CCB-DM-β-Cyd inclusion complex limited the amount of free drug available for permeation.

Increasing DM-β-Cyd and HP-β-Cyd concentration up to 5% (w/v) an enhancement of CCB Js was observed but a consistent reduction was observed in the presence of higher Cyd concentrations (10%, w/v).

CCB-DM-β-CyD samples were in solution at all considered CyD concentrations, thus the free drug was immediately available at the cutaneous surface and a more rapid permeation compared to the suspended free drug was expected. However,

the increase of CCB Js (even if low) observed at 5% (w/v) DM-β-CyD concentration with respect to 3% (w/v) concentration can not be related to the increase of the drug in solution because in both samples the drug concentration was equal. An action of the macrocycle on SC could be involved. Melnik et al. (1989), reported that SC lipids are made up of about 29% free cholesterol. Ceramides, phospholipids, free fatty acids and proteins are also present. As demonstrated by our histological studies DM-β-CyD shows an invasive action on SC, in this way it can influence the barrier properties of the skin, acting as a penetration enhancer, by a sequestration mechanism of the lipidic component of SC and/or by a disorganization of the packing order of the SC lipids.

The reduction of CCB Js and the contemporary increase of T_L observed at the highest DM-β-Cyd concentration (10%, w/v) was related to the dissociation equilibrium of the CCB-DM-β-Cyd inclusion complex. In fact, because of the complex is in equilibrium with the pure components, at higher Cyd concentrations the equilibrium was shifted towards the formation of the complex, reducing the amount of free CCB available for penetration (Masson et al., 1999).

As concerns the CCB-HP-β-Cyd system, the samples containing 3% and 5% (w/v) of macrocycle were in suspension; thus, the increase of CCB permeation was due to the progressive solubilization of the drug with increasing Cyd concentration. The sample containing the highest HP-β-Cyd concentration (10%, w/v) was in solution and the highest CCB Js was attended. However, as observed at higher DM-β-Cyd concentrations, a reduction of CCB Js with respect to the lower concentrations of the macrocycle was observed. Also in this case we can considered the interaction of CCB with HP-β-Cyd that reduced the availability of the free drug in solution. It seems that HP-β-Cyd was not able to act as a penetration enhancer for CCB permeation. Our histological analysis did not demonstrate a strong action of this macrocycle on SC, on the other hand Vollmer et al. (1994) demonstrated that HP-β-CyD is not able to interact with the lipid matrix of SC, but rather with the protein components. In addition, our previous studies on biomembrane models (Puglisi et al., 1996) showed the high affinity of DM-β-Cyd for cholesterol and phospholipids and at the same time the inability of HP-β-Cyd to interact with these components.

Histological studies demonstrated an invasive action of CCB on SC that is probably a consequence of a direct action of the drug, producing the destruction of desmosomes. This result was related to the low CCB Js observed in the percutaneous experiments; in fact, the interaction of CCB with SC could promote its accumulation at this level, limiting its passage across the epidermis. In the presence of both Cyds, CCB shows a less injurious effect on SC with respect to the free drug. Because only free components can interact with SC, this trend can be the result of the complexation between the drug and the macrocycle. Probably the interaction of CCB with SC was competitive, thus, in the presence of DM-β-Cyd the drug preferentially interacts with the macrocycle, reducing its lesive action on skin tissues. Similarly, the interaction with the drug limits the effects of Cyds on SC, reducing the injurious action of the macrocycles. In the presence of the CCB-HP-β-Cyd complex the lesions to SC were less than those observed in the presence of free CCB, but higher than those caused by HP-β-Cyd alone. It is conceivable that the absence of an inclusion complex between CCB and HP-β-Cyd and the very low K value of the external complex, allowed a higher amount of free CCB to be available for interacting with SC, with respect to the CCB-DM-β-Cyd inclusion complex.

The imbibitions of the spinosum layer observed in the presence of CCB-Cyd systems can not be justify as a result of the action of free Cyds on this stratum because they exerted no influence at this level. This effect is the probably result of the increased passage of free CCB through the epidermal layer due to the enhanced solubility of complexed CCB together with the destabilizing action on SC exerted by Cyds.

5. Conclusions

These findings demonstrate that DM-β-Cyd is able to include CCB both at the solid state and in solution, significantly increasing its water solubility. In the presence of HP-β-Cyd only a solid dispersion is obtained and in solution CCB externally interacts with this macrocycle, forming a non-inclusion complex. The in vitro results on CCB percutaneous absorption, as well as SCE carrier tolerability, prompted us to use both Cyds as potential transdermic dosage delivery systems for the localized treatment of articular diseases, obtaining a better compliance.

More ex vivo studies will be performed to evaluate the drug disposition in the various layers of SCE.

Acknowledgments

This work was financial supported by Italian MIUR. The authors are grateful to Dr. Antony Bridgewood for his revision of the language of this paper.

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